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Prevention of murine implant-associated infection by SAAP-148-loaded bioactive glass S53P4 formulation

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Introduction: Orthopedic implant-associated infections are difficult to treat due to bacterial persistence in biofilms on implant surfaces and in surrounding tissues. Bioactive glass S53P4 (BAG) is a clinically used bone graft substitute with antibacterial properties resulting from surface reactivity and dissolution-mediated changes in the local microenvironment; however, its antibacterial efficacy against established infections with a high bacterial burden is limited. In this study we therefore developed BAG putty and cream formulations loaded with synthetic antimicrobial and antibiofilm peptide SAAP-148 to enhance local antibacterial activity.

Methods: Eluates from SAAP-148-loaded and unloaded BAG putty and cream formulations were tested *in vitro* against planktonic bacteria and biofilms of *Staphylococcus aureus* and *Acinetobacter baumannii*. Antibacterial efficacy was further assessed in cadaver mouse subcutaneous implant infection model as an intermediate validation step before evaluation in an *in vivo* mouse subcutaneous implant infection model using *S. aureus*.

Results: *In vitro*, SAAP-148-loaded BAG putty and BAG cream eluates eradicated planktonic bacteria as well as young and mature biofilms of *S. aureus* and *A. baumannii*. Eluates of BAG formulations without SAAP-148 had limited bactericidal activity, with BAG cream eluates slightly more bactericidal than BAG putty eluates. The enhanced antibacterial effects of SAAP-148-loaded BAG formulations were confirmed in a cadaver mouse subcutaneous implant infection model, where *S. aureus* was eradicated both on the implant surface and in the surrounding tissue, whereas BAG-only formulations showed limited antibacterial effect. In a subsequent *in vivo* mouse subcutaneous implant infection model, SAAP-148-loaded BAG putty eradicated *S. aureus* on the implant surface and markedly reduced bacterial burden in tissue, whereas BAG putty-only failed to reduce bacterial numbers.

Conclusion: Loading SAAP-148 into BAG putty and cream enhanced BAG antibacterial and antibiofilm activity, supporting its potential as a clinically relevant adjunct to the BAG's antimicrobial activity for treating implant-associated infections.

KEYWORDS
antimicrobial peptides, bioactive glass S53P4, biofilm, orthopedic implant-associated infections, SAAP-148, *Staphylococcus aureus*

1 Introduction

Orthopedic implant-associated infections (OIAIs) remain a major clinical challenge, significantly compromising patient outcomes and increasing healthcare costs (Yi et al., 2025). These infections are particularly difficult to treat due to the formation of bacterial biofilms on implant surfaces, which protect bacteria from host immune responses and antimicrobial therapies (Zimmerli and Moser, 2012). Biofilms create a protective extracellular matrix that enables antibiotic resistance development and harbors antibiotic-tolerant persister cells, often necessitating prolonged antibiotic treatment or surgical intervention, including implant removal (Liu et al., 2024). Among the pathogens implicated in orthopedic infections, *Staphylococcus aureus* and *Acinetobacter baumannii* are of particular concern due to their strong biofilm-forming capacity and propensity for multidrug resistance (Choudhary and Shariff, 2025; Rajkumar, et al., 2026a). *S. aureus*, especially methicillin-resistant *S. aureus* (MRSA), is responsible for the majority of OIAIs due to its diverse virulence mechanisms and ability to adhere to implant surfaces (Pietrocola et al., 2022). *A. baumannii*, while primarily associated with hospital-acquired infections, is increasingly recognized in trauma-related and orthopedic infections, particularly in cases involving open fractures, contaminated wounds, orthopedic implants, and critically ill patients (Nagassar et al., 2025; Wang et al., 2025). Its remarkable ability to survive in harsh environments and rapidly acquire multidrug resistance makes it a serious clinical threat. The global public health threat posed by these pathogens is underscored by the World Health Organization (WHO), which has classified multidrug-resistant (MDR) *S. aureus* and *A. baumannii* as high-priority pathogens in both the 2017 and updated 2024 Bacterial Priority Pathogens Lists, reflecting their persistent clinical burden and the need for new therapeutic strategies (Organization, 2017; Sati et al., 2025).

Bioactive glass S53P4 (BAG) is a resorbable biomaterial clinically used in orthopedic applications, characterized by osteoconductive, osteostimulatory, and antimicrobial properties (Bigoni et al., 2019). Its antimicrobial activity is commonly attributed to physicochemical changes in the local microenvironment resulting from its surface reactivity, thereby creating conditions unfavorable for bacterial survival (Rajkumar, et al., 2026a; Rajkumar, et al., 2026b; Zhang et al., 2010). S53P4 BAG is commercially available as granules (0.5–0.8 and 1.0–2.0 mm), which are clinically used for bone defect filling and infection management, and as a putty formulation intended primarily as a bone void filler (Thijssen et al., 2020). The putty consists of BAG granules combined with a polymeric binder composed of polyethylene glycol (PEG) and glycerol (Thijssen et al., 2020). For infection control in patients, BAG granules, either alone or in combination with BAG putty, are typically used together with systemic antibiotics (Nguyen et al., 2025). In an *in vivo* model of infected non-union bone, BAG putty alone failed to eradicate infection in the absence of systemic antibiotic therapy (Freischmidt et al., 2022), indicating that BAG putty may be insufficient as a standalone treatment for infection control. Fine BAG powder, consisting of smaller glass particles (≤45 μm), exhibits enhanced antimicrobial activity compared with commercial granules (Gonzalez Moreno et al., 2020; Rajkumar, et al., 2026a). This effect has been attributed to its high specific surface area-to-volume ratio, which promotes rapid ion release and increases local ion concentrations (Muller et al., 2024; Rajkumar, et al., 2026a). To utilize these properties, a BAG cream formulation was developed by combining BAG powder with a PEG/glycerol binder, which has potent broad-spectrum microbicidal activity against planktonic bacteria and biofilms *in vitro* (Rajkumar, et al., 2026a). However, in a cadaver mouse bone-defect model, BAG cream only inhibited the growth of *S. aureus*, rather than achieving complete eradication (Rajkumar, et al., 2026a). Together, these findings suggest that BAG putty and BAG cream may benefit from incorporation of additional antimicrobial agents to enhance infection prevention and treatment in challenging bacterial settings.

Antimicrobial peptides (AMPs), such as synthetic antimicrobial and antibiofilm peptide SAAP-148, have emerged as potent candidates for combination with antibiotics and biomaterials platforms (Atif et al., 2024; Bouhrour et al., 2023; Koppen et al., 2019; Schmitz et al., 2023) due to their broad-spectrum antimicrobial activity, rapid mechanism of action, and consequently low likelihood of resistance development (de Breij et al., 2018). SAAP-148 is effective against a broad spectrum of microorganisms, including both Gram-positive and Gram-negative bacteria and their MDR strains, and has demonstrated strong capacity to kill bacteria in biofilms and eradicate murine skin infection (Atif et al., 2024; de Breij et al., 2018). Importantly, SAAP-148 is compatible with conventional antibiotics and can even exhibit synergistic activity, such as with teicoplanin (Koppen et al., 2019). This allows SAAP-148 to be integrated with standard antibiotic prophylactic regimens such as vancomycin used in orthopedic surgery (Bouhrour et al., 2023; Koppen et al., 2019; Lennard et al., 2025; van Gent et al., 2022). Incorporating SAAP-148 into BAG-based formulations may enable a dual-action approach at the site of infection by combining the intrinsic antimicrobial properties of BAG with the potent microbicidal and antibiofilm activity of SAAP-148. When loaded into BAG putty or BAG cream this may facilitate local release of SAAP-148, potentially achieving high local concentrations that could enhance the antibacterial activity of BAG and promote infection clearance.

In this study, we investigate the bactericidal and biofilm-killing properties of SAAP-148-loaded S53P4 BAG putty and cream against clinically relevant MDR *S. aureus* and MDR *A. baumannii*. We evaluate these formulations through three complementary approaches: (1) *in vitro* testing of BAG putty and BAG cream eluates, with and without SAAP-148; (2) a cadaver mouse subcutaneous implant infection model comparing BAG putty, BAG cream, and their respective SAAP-148-loaded formulations; and (3) an *in vivo* mouse subcutaneous implant infection model comparing the infection control by BAG putty and SAAP-148-loaded BAG putty.

2 Materials and methods

2.1 Bioactive glass S53P4 putty and cream and their SAAP-148-loaded formulations

SAAP-148 (≥98% purity) was obtained from Synpeptide (China). Four S53P4 bioactive glass (BAG)-based formulations

were used: BAG putty, BAG cream, BAG putty with 2% (*w/v*) SAAP-148 ("SAAP-BAG putty"), and BAG cream with 2% (*w/v*) SAAP-148 ("SAAP-BAG cream"). The commercially available Bonalive® putty was used, which consisted of 60% (*w/w*) granules (particle size 500–800 μm) and 40% (*w/w*) binder. The BAG cream formulation consisted of 50% (*w/w*) BAG S53P4 powder (particle size ≤ 45 μm) and 50% (*w/w*) binder composed of different polyethylene glycol (PEG) species and glycerol. Both BAG putty and BAG cream were supplied by Bonalive Ltd (Turku, Finland), which also prepared the corresponding SAAP-148-loaded formulations. The SAAP-148 concentration of 2% (*w/v*) was based on a previous study (de Breij et al., 2018). SAAP-BAG putty was prepared by heating the ready-made S53P4 BAG putty formulation to 60 °C under continuous stirring, adding SAAP-148 to obtain a final peptide concentration of 2% (*w/v*), and cooling the mixture to room temperature while stirring to ensure homogeneous distribution of the peptide within the putty matrix. The thermal stability of SAAP-148 at 60 °C was confirmed by incubating pure SAAP-148 at 60 °C for 30 min, which did not reduce its bactericidal activity compared with unheated SAAP-148 (results not shown). For SAAP-BAG cream, SAAP-148 was first added to the PEG/glycerol binder before incorporation of the fine BAG powder to facilitate homogeneous mixing before the formulation became highly viscous.

2.2 Bacteria

Before each experiment, MDR *S. aureus* LUH14616 and *A. baumannii* RUH875 were prepared by incubating 1 to 3 colonies in 5 mL tryptic soy broth (TSB; BD Difco, United States) at 37 °C with shaking at 120 rpm overnight. The next day, 100 μL of the overnight culture was sub-cultured in 5 mL fresh TSB and incubated for 3 h at 37 °C to reach mid-logarithmic growth phase.

Mid-log phase cultures were washed twice with RPMI 1640 medium (20 mM HEPES, L-glutamine, without sodium bicarbonate; Sigma-Aldrich, United Kingdom) and resuspended to 1×10^7 colony forming units (CFU)/mL in RPMI, based on the optical density at 620 nm. From this suspension, 100 μL (1×10^6 CFU) or 10 μL (1×10^5 CFU) were used as inocula, unless indicated otherwise.

2.3 Preparation of eluates

Eluates of BAG putty, BAG cream, SAAP-BAG putty, and SAAP-BAG cream were produced following a previously described procedure (Rajkumar, et al., 2026a). Briefly, 200 mg of each formulation was added to individual wells of a 24-well plate, followed by the addition of 1 mL of RPMI, and allowed to elute for either 5, 10, 30 min, 1, 2, 4, or 24 h at 37 °C and 120 rpm. Subsequently, the RPMI medium, now containing the eluted components from BAG putty or cream with or without SAAP-148, was collected and centrifuged at 20,800 RCF for 10 min to remove BAG particles. The supernatants were collected and referred to as "eluates". The eluates were then used either undiluted (100%) or as serial 2-fold dilutions in RPMI for antimicrobial activity testing. The pH was measured for undiluted and 1:4-diluted eluates using a pH sensor (Eutech Instruments, Waterproof pH Testr30). RPMI medium incubated under the same conditions served as the control.

2.4 Bactericidal activity of SAAP-148-loaded BAG putty and BAG cream eluates

The bactericidal activity of the eluates of BAG putty, BAG cream, SAAP-BAG putty, and SAAP-BAG cream was quantified by adding either 100 μl (containing 1×10^6 CFU) or 10 μl (containing 1×10^5 CFU) of *S. aureus* or *A. baumannii* bacterial inoculum to 900 μl or 90 μl of undiluted or diluted eluate in 24-well or 96-well polypropylene microtiter plates (Costar), resulting in a final bacterial concentration of 1×10^6 CFU/mL. The plates were incubated at 37 °C and 120 rpm for 24 h. The incubations were then serially ten-fold diluted, and from each dilution, four 10 μl droplets were plated on blood agar plates (Oxoid, United Kingdom). Plates were incubated overnight at 37 °C, and the number of CFU were counted. All conditions were tested in triplicate. Limit of detection for bactericidal activity is 25 CFU/mL.

The lethal concentration causing 99.9% killing of bacteria (LC99.9; i.e., a 3-log reduction in CFU compared with the inoculum) was determined for pure SAAP-148 for both strains, along with the eluate dilutions achieving ≥99.9% killing. The highest dilution achieving ≥99.9% killing was identified for each eluate. Based on the LC99.9 concentration of pure SAAP-148 and the eluate dilutions, the SAAP-148-equivalent concentration in the eluates was calculated.

2.5 Biofilm-killing activity

The biofilm-killing activity of 2 h eluates of all formulations was assessed against 24 h (young) and 72 h (mature) biofilms of *S. aureus* or *A. baumannii* (1×10^8 CFU/mL in RPMI +2% glucose medium). A total of 100 μl of each suspension was added to two 96-well plates, one "before-treatment" and one "after-treatment" plate. After static incubation for 24 h and 72 h at 37 °C, the medium was carefully removed, and biofilms were gently washed with 100 μL of PBS to remove non-adherent cells. To determine baseline CFU numbers, biofilms in the "before-treatment" plate were resuspended in 100 μL PBS and dispersed by sonication for 5 min at 37 kHz (Elma Transsonic 460) in sealed plates. To assess the effect of eluate treatment on biofilms, 100 μl eluate or control medium was added to the biofilms in the "after treatment" plate, which was then incubated for 2, 8 and 24 h at 37 °C. Following this treatment, biofilms were gently washed to remove planktonic bacteria, resuspended in 100 μL PBS containing 0.025% sodium polyanethol sulphonate (SPS), and sonicated for 5 min as described above to disperse the biofilms. The suspensions were serially diluted and plated on blood agar for CFU quantification.

2.6 Bactericidal activity of SAAP-148-loaded BAG putty and BAG cream in a cadaver mouse subcutaneous implant infection model

The cadaver mouse subcutaneous implant infection model was performed as previously described (Riool et al., 2014), with modifications to incorporate a BAG-based treatment approach. Briefly, the back of the C57BL/6 mice cadavers were shaved using an electric razor, followed by skin disinfection with 70% ethanol. Then, two solid medial grade (ISO 5832/11)

subcutaneous titanium implants (10 mm × 4 mm × 1 mm, with a slit (Riool et al., 2014)); AO Research Institute, Davos, Switzerland) were placed in the dorsal region. On each side, an incision of 0.4 cm was made 1 cm lateral to the spine. Each implant was pre-inoculated with *S. aureus* by applying two 5 µL droplets of bacterial suspension (4×10^5 CFU per droplet; total 8×10^5 CFU per implant) to the left and right of the central slit of the implant (Riool et al., 2014) and briefly air-dried just before implantation. Subsequently, approximately 50 mg of BAG formulation was applied per implant within the subcutaneous pocket. Four treatment groups were evaluated: BAG putty, BAG cream, SAAP-BAG putty, and SAAP-BAG cream, along with an inoculum-only control (n = 4 implants per group, i.e. 2 cadavers per group with 2 implants each). Skin incisions were closed with a single 6–0 Vicryl stitch (Vicryl, Ethicon, Raritan, NJ, United States), and tissue adhesive (Histoacryl) was applied to cover the suture. Following overnight incubation at room temperature, standardized circular biopsies with a diameter of 12 mm were collected from each implantation site, including the skin, subcutaneous tissue, and implant. Implants were then separated from the tissue and processed separately for quantitative culture, including neutralization of SAAP-148 activity as described previously (Riool et al., 2014).

2.7 *In vivo* bactericidal activity of SAAP-148-loaded BAG putty in a subcutaneous implant infection model

The mouse study was approved by the Animal Welfare Body of the Amsterdam UMC (location AMC), Amsterdam, The Netherlands (study number: SP2600067; 10 April 2026). Specific pathogen-free C57BL/6J OlaHsd immunocompetent female mice (Envigo RMS B.V., Horst, The Netherlands), aged 8–10 weeks, were used. Groups of 9 mice each receiving 2 pre-inoculated subcutaneous implants with either non-loaded BAG putty or SAAP-BAG putty (n = 18 implants per group). BAG putty was selected for the *in vivo* study rather than BAG cream, since BAG putty is approved for human use.

The experiment was performed according to the procedure described in detail earlier (Riool et al., 2014). Briefly, mice were anesthetized with 2% isoflurane (Pharmachemie, Haarlem, The Netherlands) in oxygen in a laminar flow cabinet. Followed by shaving the back of the mice and skin disinfection with 70% ethanol, subcutaneous injection of buprenorphine (Temgesic, RB Pharmaceuticals Limited, 0.05 mg/kg) for pain control was administered 30 min prior to surgery. The subcutaneous implants were pre-inoculated with *S. aureus* (8×10^5 CFU per implant) as described for the cadaver model. The implants were inserted into subcutaneous pockets, after which approximately 50 mg of BAG putty or SAAP-BAG putty was applied onto and around each implant using sterile instruments. The incisions were closed with a single 6–0 Vicryl stitch and covered with tissue adhesive. Mice were housed individually in individually ventilated cages, with one mouse per cage, and provided sterile food and water *ad libitum*. They were weighed before surgery and again prior to euthanasia.

One day after implantation, mice were anesthetized with 2% isoflurane in oxygen and received buprenorphine 30 min before explantation. From each implantation site, the implant and surrounding tissue were collected. Half of the collected tissue was stored at −80 °C for future analyses, while the remaining tissue and implant samples were processed for quantitative culture as described previously (Riool et al., 2014). Mice were then euthanized by cervical dislocation.

2.8 Statistical analysis

Statistical analyses were performed using GraphPad Prism 10 (GraphPad, San Diego, CA, United States). Bactericidal activity and cadaver experiments were evaluated using two-way analysis of variance (ANOVA) with Tukey's *post hoc* test. Biofilm-killing experiments were analyzed using one-way ANOVA with Tukey's *post hoc* test. For *in vivo* experiments, comparisons of log CFU numbers between the two groups were conducted using a two-tailed Mann-Whitney rank sum test. Differences in categorical variables were assessed using Fisher's exact test. For all tests, p-values ≤ 0.05 were considered significant (*p = 0.01-0.05, **p = 0.001-0.01, ***p = 0.0001-0.001, ****p < 0.0001).

3 Results

3.1 BAG cream has higher bactericidal activity than BAG putty

The bactericidal activity of BAG putty and BAG cream was compared using eluate testing. Eluates collected at 2 and 24 h were tested in undiluted (100%) and diluted (25%) form. Alkalinity is considered an important factor in BAG antimicrobial activity and therefore the pH of BAG eluates were measured before bacterial exposure. The pH of undiluted BAG putty and BAG cream eluates was 10.5 and 10.9 at 2 h, respectively, and 10.5 and 10.2 at 24 h, whereas the corresponding 25% eluates had pH values of 9.6 and 9.4 at 2 h, and 9.3 and 9.1 at 24 h (Figure 1A).

Undiluted BAG cream eluates collected at both time points completely eradicated the inoculum of *S. aureus* and *A. baumannii*, whereas undiluted BAG putty eluates consistently achieved an approximately 3-log reduction in bacterial CFU compared to the inoculum control (Figures 1B,C). The decrease in killing with dilution of the eluates to 25% indicated that BAG eluates lost bactericidal strength when diluted (Figures 1B,C). Overall, these results indicate that BAG eluates are strongly antibacterial, but their bactericidal activity is markedly reduced upon 25% dilution. These findings demonstrate that incorporation of an additional antimicrobial component, such as SAAP-148, can further enhance the bactericidal activity of BAG formulations and promote complete bacterial eradication.

3.2 Eluates of SAAP-148-loaded BAG putty and cream have enhanced bactericidal activity

We next evaluated whether incorporation of SAAP-148 into BAG formulations would enhance the bactericidal activity of BAG putty and BAG cream eluates. Eluates were prepared from BAG putty and BAG cream either with or without SAAP-148 and subsequently diluted to 25%. The pH values of diluted eluates were alkaline, measuring 9.6 for BAG putty and 9.7 for BAG

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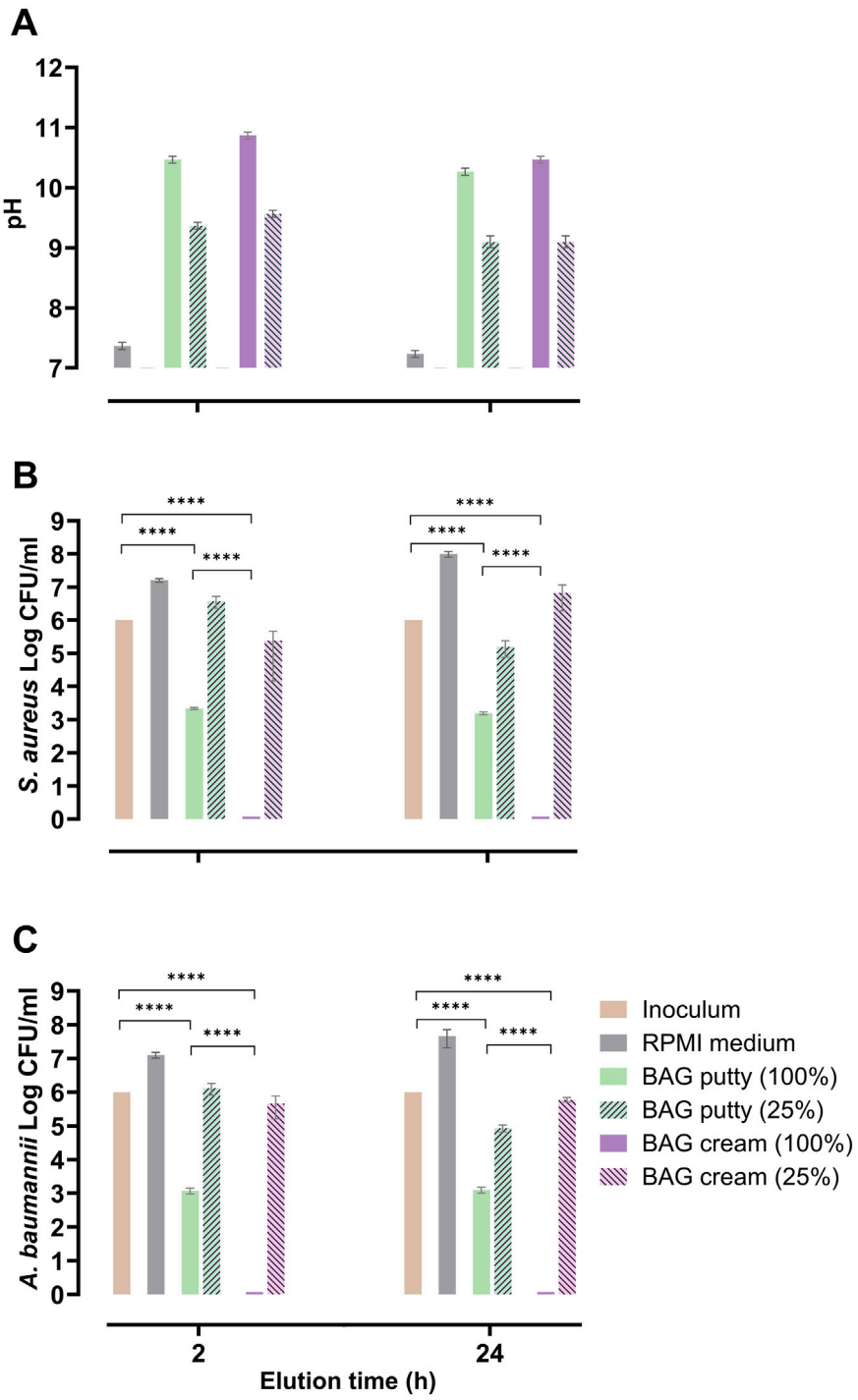


FIGURE 1
Bactericidal activity and pH of BAG putty and BAG cream eluates collected after 2 and 24 h. **(A)** pH values of BAG eluates and their 25% dilutions. **(B,C)** Bactericidal activity of undiluted (100%) and diluted (25%) eluates against *Staphylococcus aureus* **(B)** and *Acinetobacter baumannii* **(C)**. Bars indicate mean ± standard deviation (SD) from one experiment with three technical replicates (n = 3).

cream eluates at 2 h, and decreasing to 9.0 and 9.2 at 24 h, respectively. In the presence of SAAP-148, a slight reduction in pH was observed. The pH values of SAAP-BAG putty and SAAP-BAG cream, were 9.3 and 9.6 at 2 h, respectively, and 8.9 and 9.1 at 24 h (Figure 2A). Consistent with the findings in Figure 1, diluted eluates of BAG putty and cream alone had minimal or no bactericidal activity. However, upon addition of SAAP-148, 25% eluates of both BAG putty and BAG cream completely killed *S. aureus* and *A. baumannii* inocula (Figures 2B,C). Overall, incorporation of SAAP-148 markedly enhanced the bactericidal activity of diluted BAG putty and BAG cream eluates while having only a minor effect on the BAG-associated increase in pH.

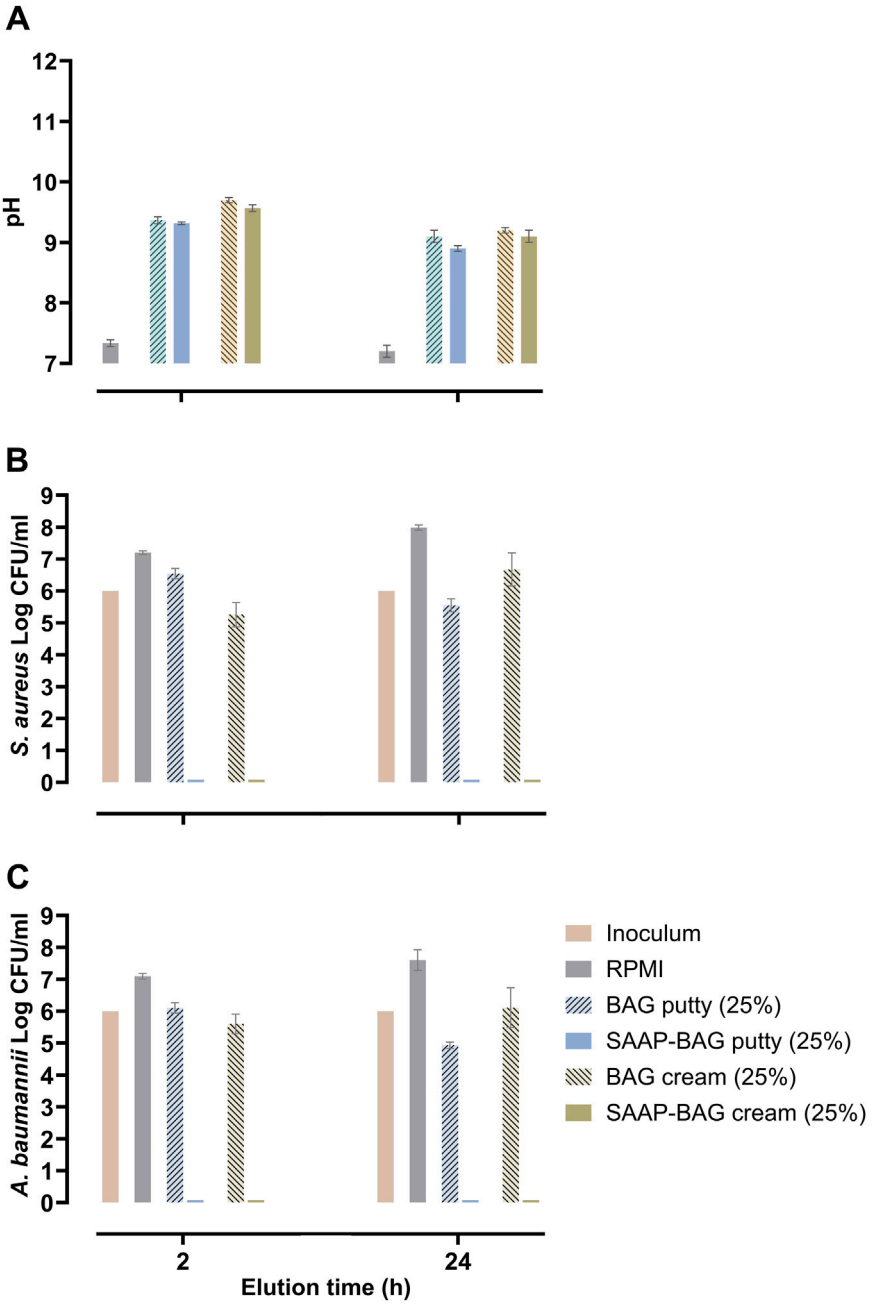


FIGURE 2
Bactericidal activity and pH of 25% diluted BAG putty and BAG cream eluates with and without SAAP-148 at 2 h and 24 h. **(A)** pH values of diluted BAG eluates with and without SAAP-148. **(B,C)** Bactericidal activity of diluted eluates with and without SAAP-148 against *Staphylococcus aureus* **(B)** and *Acinetobacter baumannii* **(C)**. Bars indicate mean ± SD from one experiment, with three technical replicates (n = 3).

3.3 Rapid release of bactericidal components of SAAP-148-loaded BAG putty and BAG cream

To determine the elution kinetics and bactericidal activity of SAAP-148-loaded BAG formulations, eluates collected at different time points were assessed for their bactericidal activity using the LC99.9 assay against *S. aureus* and *A. baumannii*.

The pH of undiluted BAG eluates increased markedly, within 5 min, to alkaline pH levels, from initial neutral pH in the RPMI control, and remained alkaline throughout the elution period of 24 h, with BAG cream eluates having slightly higher pH values than BAG putty eluates (Figure 3A). Addition of SAAP-148 caused only a minor lowering of the pH, which remained stable over time.

BAG putty and BAG cream eluates without SAAP-148 showed limited bactericidal activity against both bacterial species, reaching ≥99.9% killing only with eluates collected from 2 h onwards. BAG cream eluates showed slightly higher bactericidal activity than BAG putty eluates (Figure 3B). In contrast, eluates from SAAP-BAG putty and SAAP-BAG cream showed rapid and

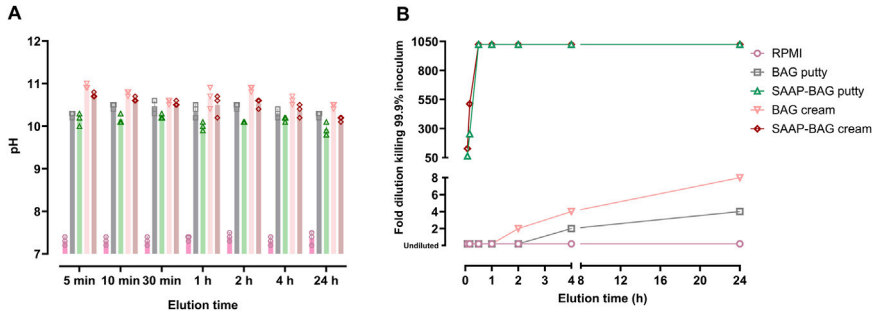


FIGURE 3
pH of undiluted (100%) eluates of BAG putty, SAAP-BAG putty, BAG cream, and SAAP-BAG cream and bactericidal potency of eluates expressed as the maximum fold dilution killing >99.9% killing of the test bacteria, and **(A)** pH values of undiluted eluates collected over time. **(B)** Bactericidal potency of eluates collected over time, expressed as the maximum fold dilution killing >99.9% killing of the bacterial inoculum. Identical fold-dilution values and bactericidal activity were observed against *Staphylococcus aureus* and *Acinetobacter baumannii*; therefore, the *S. aureus* data are shown as representative results. Data are shown as individual values with mean ± SD from one experiment with three technical replicates (n = 3).

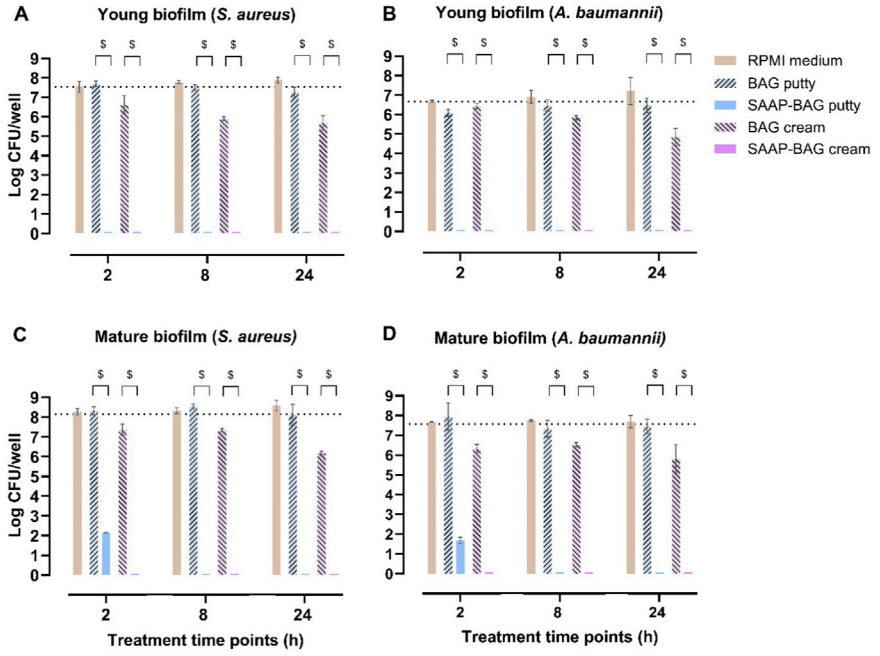


FIGURE 4
Biofilm-killing activity of 2 h eluate of BAG putty, SAAP-BAG putty, BAG cream, and SAAP-BAG cream eluates against 24 h (young) and 72 h (mature) biofilms. *Staphylococcus aureus* biofilms are shown in **(A)** and **(C)**, and *Acinetobacter baumannii* biofilms in **(B)** and **(D)**. Biofilms were treated with eluates for 2, 8 and 24 h. Bars indicate mean ± SD of six technical replicates (n = 6) from one experiment. Statistical comparisons were performed against the starting biofilm CFU numbers and between each BAG-only eluate and its corresponding SAAP-BAG formulation eluate. For clarity, only the statistical comparisons between BAG-only and SAAP-BAG formulation eluates are shown in the graphs ($, $p \leq 0.0001$), whereas comparisons with the starting biofilm CFU numbers are described in the results text.

markedly enhanced bactericidal activity compared with their respective non-loaded BAG formulations. Already after 5 min of elution, ≥99.9% killing was detected at substantially higher fold dilutions compared with eluates of non-loaded BAG. SAAP-BAG putty and SAAP-BAG cream showed bactericidal activity at 64-fold and 128-fold dilutions at 5 min, at 256-fold and 512-fold dilutions after 10 min, and up to 1024-fold dilution from 30 min up to 24 h (Figure 3B). This indicated rapid release of SAAP-148 from both BAG formulations (Figure 3B). SAAP-BAG cream eluates at the early time points had slightly higher activity than SAAP-BAG putty eluates, as they could be diluted further, but from 30 min onwards the bactericidal activity of both the formulations was the same.

The eluate fold dilutions killing 99.9% of bacteria were used to estimate the release of SAAP-148-associated bactericidal activity from SAAP-148-loaded BAG putty and BAG cream. Pure SAAP-148 showed an LC99.9% at 0.94 μM against both bacterial species. For each eluate, the highest fold dilution that still achieved 99.9% bacterial killing was multiplied by 0.94 μM to calculate the

equivalent SAAP-148 concentration. Based on this calculation, the equivalents SAAP-148 concentrations released from SAAP-BAG putty and SAAP-BAG cream were 60.16 and 120.32 μM at 5 min, 240.64 and 481.28 μM at 10 min, and increased to 962.56 μM from 30 min onwards for both formulations. These values should be interpreted as SAAP-148-equivalent bactericidal concentrations rather than pure SAAP-148 concentrations, since bacterial killing may result from the combined activity of SAAP-148 and antimicrobial components released from BAG.

A checkerboard assay performed as previously described for SAAP-148 and antibiotics (Koppen et al., 2019) showed no synergistic or antagonistic interaction between SAAP-148 and BAG (data not shown). Therefore, the enhanced bactericidal activity of SAAP-BAG is attributed to the combined antibacterial effects of SAAP-148 and BAG rather than true synergism.

Overall, these results show that incorporation of SAAP-148 enhances the bactericidal activity of BAG eluates, enabling ≥99.9% bacterial killing rapidly and at markedly higher fold dilutions than BAG alone.

3.4 SAAP-148 loaded BAG putty and BAG cream eradicate bacterial biofilms

The biofilm-killing activity of BAG putty, BAG cream, SAAP-BAG putty, and SAAP-BAG cream was assessed by evaluating eluates collected at 2 h against 24 h (young) and 72 h (mature) biofilms (Figure 4). BAG cream eluates significantly reduced *S. aureus* and *A. baumannii* young and mature biofilm CFU numbers compared with starting levels. This reduction was significant at all treatment time points, except for *A. baumannii* at 2 h. In contrast, BAG putty eluates showed no significant reduction at the 2, 8, or 24 h treatment time points. Similar to the findings for planktonic bacteria, BAG cream eluates were more effective in reducing biofilm CFU numbers than BAG putty eluates.

SAAP-BAG putty and SAAP-BAG cream eluates eradicated the 24 h biofilms after 2, 8, and 24 h of treatment. For 72 h biofilms, 2 h of treatment resulted in an approximately 6-log reduction in biofilm CFU numbers for both *S. aureus* and *A. baumannii*, whereas 8 and 24 h of treatment fully eradicated the biofilm bacteria. These results indicate that while BAG eluates alone exhibit limited to moderate biofilm-killing activity, incorporation of SAAP-148 resulted in complete biofilm eradication, highlighting a substantial enhancement in activity against biofilms of both Gram-positive and Gram-negative bacteria.

3.5 SAAP-148-loaded BAG putty and cream completely eradicate *Staphylococcus aureus* from implants and surrounding tissue in a cadaver subcutaneous infection model

The cadaver mouse subcutaneous implant infection model was used to evaluate the applicability and bactericidal activity of BAG putty, SAAP-BAG putty, BAG cream, and SAAP-BAG cream against *S. aureus*. All four formulations were easy to apply and remained localized around the implant. On the infected implant surface, BAG putty and BAG cream achieved approximately 2- and 3-log reductions in CFU counts, whereas SAAP-BAG putty and SAAP-BAG cream achieved complete bacterial eradication

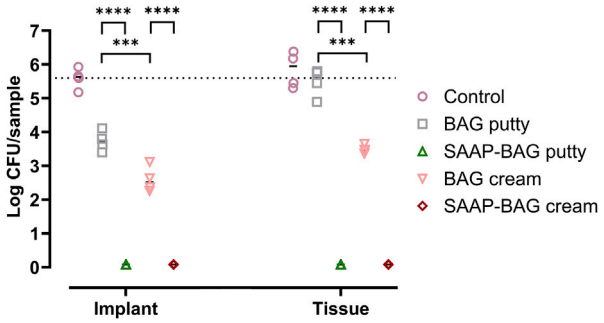


FIGURE 5
Bactericidal activity after 1 day of *Staphylococcus aureus* inoculation with BAG putty, SAAP-BAG putty, BAG cream, and SAAP-BAG cream in a cadaver mouse subcutaneous implant infection model. Results are shown as log CFU per sample for implants and surrounding tissue (n = 4 samples, derived from 2 cadavers), with each data point representing an individual sample and the horizontal line indicating the median. The horizontal dotted line indicates the inoculum size. In the graphs, statistical significance is shown only for the comparisons between BAG-only formulations and their corresponding SAAP-148-loaded formulations. Comparisons of each treatment group with the untreated control are described in the results text.

(Figure 5). Both SAAP-148-loaded BAG formulations showed a statistically significant reduction in CFU numbers compared with their respective BAG-only counterparts. In the surrounding tissue, BAG putty alone did not significantly reduce bacterial counts compared with the control. BAG cream achieved an approximately 2-log reduction in CFU counts, while both SAAP-148-loaded BAG formulations achieved complete eradication (Figure 5). A slight increase in CFU was observed in the tissue of the untreated control compared with the initial inoculum (4×10^5 CFU) (Figure 5).

These results demonstrate that while BAG formulations alone exhibit limited (putty) to moderate bactericidal activity (cream) in a cadaver subcutaneous tissue environment, incorporation of SAAP-148 led to complete bacterial eradication on both implant surface and in the surrounding tissue.

3.6 SAAP-148-loaded BAG putty eradicates implant-associated *Staphylococcus aureus* and markedly reduces surrounding tissue infection *in vivo*

The *in vivo* mouse subcutaneous implant infection model was used to assess the bactericidal activity of BAG putty and SAAP-BAG putty on infected titanium implants and surrounding tissue 1 day after infection. On the implant surface as well as in the surrounding tissue, all the samples of the BAG putty group were culture positive (18/18; 100%). SAAP-BAG putty eradicated all bacteria on the implant surface, while 11 of 18 tissue samples were culture negative (61%) and 7 were culture positive (39%) (p = 0.0001). In the culture-positive samples, CFU numbers were significantly reduced by an average of approximately 2 log compared with the inoculum ($p \leq 0.0001$) (Figure 6). No macroscopic signs of inflammation or abnormal tissue appearance were observed upon visual examination. Thus, loading BAG putty with SAAP-148

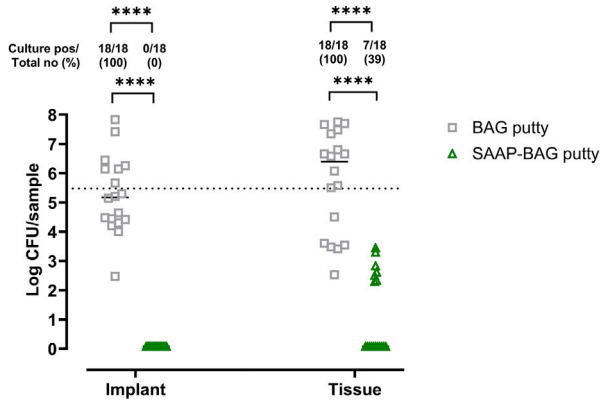


FIGURE 6
Bactericidal activity of BAG putty and SAAP-BAG putty against *Staphylococcus aureus* in a mouse subcutaneous implant infection model 1 day after infection. Titanium implants were pre-inoculated with bacteria and implanted subcutaneously, after which they were treated with BAG putty or SAAP-BAG putty. Results are shown as log CFU per sample retrieved from implants and surrounding tissue (n = 18 implants and n = 18 tissue samples, obtained from 9 mice), with each data point representing an individual sample, the horizontal line indicating the median, and the dotted line indicating the inoculum size. The frequencies (and percentages) of culture-positive samples are indicated above the graph. Statistical analysis of culture-positive samples and log CFU values was performed using Fisher's exact test and Mann–Whitney test, respectively.

strongly increased its microbicidal capacity, eradicating bacteria from the implant surface and markedly reducing the bacterial burden in the surrounding tissue.

4 Discussion

This study demonstrates that loading S53P4 bioactive glass (BAG) putty or cream formulations with the SAAP-148 antimicrobial peptide substantially enhances their antibacterial and antibiofilm activity. *In vitro*, BAG cream eluates completely eradicated both planktonic *S. aureus* and *A. baumannii*, but lost most of their activity already at 4-fold dilution, and did not completely eradicate the number of viable bacteria in their respective biofilms. BAG putty eluates achieved approximately a 3-log reduction in planktonic bacterial counts, but showed limited biofilm-killing activity. Incorporation of SAAP-148 into either of the BAG formulations enhanced their bactericidal potency, achieving complete eradication of planktonic bacteria and biofilms of *S. aureus* and *A. baumannii in vitro*. These enhanced antibacterial effects were confirmed in a cadaver mouse subcutaneous implant infection model, in which SAAP- BAG putty and SAAP-BAG cream achieved complete bacterial eradication both on implant surfaces and in the surrounding tissue, in contrast to the BAG formulations without SAAP-148, which only partially reduced the numbers of CFU. In a subsequent *in vivo* mouse subcutaneous implant infection model, SAAP-148-loaded BAG putty achieved complete bacterial clearance on implant surfaces after 1 day, whereas BAG putty alone failed to reduce numbers of bacterial CFU. Taken together, these findings establish SAAP-148-loaded BAG formulations as effective dual-action local antimicrobial and provide proof-of-concept for their further evaluation in the prevention and treatment of orthopedic implant-associated infections.

In clinical practice, BAG S53P4 granules and putty are generally used as part of a combined antimicrobial treatment strategy, including surgical debridement and systemic antibiotics selected based on bacterial culture and susceptibility testing (Nguyen et al., 2025; Van Vugt et al., 2021). A prospective multicenter study evaluating 78 patients with chronic cavitary long bone osteomyelitis reported an overall infection eradication rate of 85%, with a one-year infection-free survival of 89%, achieved through debridement, BAG implantation, and systemic antibiotic therapy (Lindfors et al., 2026). A recent systematic review and meta-analysis including 28 observational studies and case series, encompassing 1,122 patients with osteomyelitis, reported a pooled infection eradication rate of 88.1% with BAG S53P4. Most patients received systemic antibiotics, with treatment protocols generally combining surgical debridement, S53P4 implantation, and systemic antimicrobial therapy. These findings support the clinical use of BAG S53P4 as an adjunctive local treatment rather than a complete replacement for systemic antibiotics (Nguyen et al., 2025). A retrospective study on mastoid and epitympanic obliteration reported a 4% infection rate when BAG granules were combined with antibiotics, compared to 22% when used without antibiotics (Alciato et al., 2022). Similarly, a prospective clinical study in a low-resource setting reported successful infection control in only 38% of patients as assessed at 24 months after debridement and S53P4 granule implantation, whereas infection persisted or recurred in 61.5% (Geurts et al., 2018). These results were likely influenced by major implementation-related constraints, including limited diagnostic resources, inconsistent availability and quality of systemic antibiotics, shortened antibiotic treatment, and incomplete follow-up. Thus, the results of this study are difficult to interpret, but seem to show the limitations of BAG-based treatment when systemic antibiotic therapy cannot be reliably implemented, rather than the efficacy of BAG under standard antibiotic protocols. The reliance on systemic antibiotics for infection clearance carries important clinical consequences. Prolonged antibiotic courses are associated with systemic toxicity, selection for resistance, and poor tissue penetration at poorly vascularized infection sites (Polyzou et al., 2026; Tao et al., 2021; Wang et al., 2023). These limitations are compounded by the global rise of MDR pathogens such as MRSA and MDR *A. baumannii*, both classified as high-priority pathogens (Sati et al., 2025). We therefore combined BAG putty and cream with the antimicrobial peptide SAAP-148.

The potent bactericidal activity observed with SAAP- BAG putty and SAAP-BAG cream is consistent with previous evidence showing that SAAP-148 retains activity under physiologically complex conditions and displays strong *in vivo* efficacy, with topical 2% SAAP-148 in hypromellose ointment markedly reducing or eradicating acute and established *S. aureus* and *A. baumannii* biofilm infection in murine abraded skin following a 4 h treatment period (de Breij et al., 2018). We used a 2% addition of SAAP-148 to the BAG cream and putty as well and observed high efficacy. A limitation of our study is that SAAP-148 concentrations could not directly be quantified; therefore, the calculated SAAP-148-equivalent bactericidal concentrations do not represent directly measured peptide concentrations or release kinetics and hence

they are designated as 'SAAP-148 equivalent bactericidal concentrations.

Crucially, the membrane-disruption mechanism of SAAP-148 operates independently of bacterial metabolic activity, enabling it to kill metabolically inactive persister cells and biofilm-embedded micro-organisms that are intrinsically resistant to conventional antibiotics (Adelaide et al., 2023; Kim, 2026; Yan and Bassler, 2019). This is of particular relevance because dissolution of BAG S53P4 generates an alkaline microenvironment with elevated pH. Although this alkalinity exceeds physiological tissue pH, S53P4 BAG is clinically used and its local alkaline response is an established consequence of its dissolution. Moreover, no adverse clinical signs were observed during the 1-day follow-up in the present *in vivo* study. Elevated environmental pH has been implicated as a stress factor influencing bacterial persistence, and pH-induced stress has been suggested as a potential cue for persister formation (Karki et al., 2020). Therefore, although BAG-induced alkalinity has not been directly shown to induce *S. aureus* persisters, it represents a pH-related stress condition which requires an incorporated antimicrobial agent to remain active and capable of eradicating persisters (Scheper et al., 2021). The present finding that SAAP-148 retained full bactericidal and biofilm-killing activity at the alkaline pH of BAG eluates supports its suitability for incorporation into BAG-based formulations.

The synergistic potential of SAAP-148 with other antimicrobial agents further strengthens the rationale for its integration into clinical infection management (Chi et al., 2025). SAAP-148 showed synergism with teicoplanin, a clinically used glycopeptide antibiotic against *S. aureus*, and did not interact with other classes of antibiotics, indicating compatibility with conventional antibiotic treatment (Koppen et al., 2019). Moreover, SAAP-148 has been shown to act synergistically with halicin, originally a non-antibiotic compound later identified and repurposed as a potential antibiotic, against MDR strains of *E. coli* and *S. aureus* in 3D human skin infection models (Lennard et al., 2025; van Gent et al., 2022). These findings support a local infection prevention strategy in which SAAP-148 provides rapid antibacterial protection, while the BAG formulation itself contributes antibacterial activity at later time points, from approximately 2 h onward. This local dual-action effect could be combined with systemic antibiotic prophylaxis, supported by the reported compatibility of SAAP-148 with conventional antibiotics.

Previous studies have shown that BAG granules and powder can reduce *S. aureus* biofilm CFU on titanium surfaces *in vitro*, with BAG powder consistently achieving greater reductions than the larger granules (Coraca-Huber et al., 2014). In the present study, 2 h eluates from BAG putty, BAG cream, SAAP-BAG putty, and SAAP-BAG cream were evaluated against 24-h (young) and 72-h (mature) biofilms after treatment for 2, 8, or 24 h .Eluates from BAG cream, which contains powder, were more effective than eluates of BAG putty, which contains granules. BAG cream eluates achieved an approximately 1–2 log reduction in both young and mature biofilm-associated *S. aureus* and *A. baumannii*, although complete biofilm eradication was not achieved. These data indicate that neither BAG putty nor BAG cream alone is sufficient for complete biofilm eradication. In contrast, SAAP-BAG putty and SAAP-BAG cream eluates eradicated 24-h biofilms after 2, 8, and 24 h of treatment. Against 72-h biofilms, both SAAP-BAG eluates caused an approximately 6-log reduction after 2 h of treatment and eradicated the biofilms after 8 or 24 h. These findings show that SAAP-148 substantially enhanced the activity of both BAG formulations against young and mature *S. aureus* and *A. baumannii* biofilms, consistent with our prior work demonstrating that SAAP-148 fully eradicates MDR biofilms on *ex vivo* human skin and in murine wound models. (de Breij et al., 2018). A limitation of this biofilm model is the use of polystyrene rather than titanium surfaces as representation of actual orthopedic implants, warranting future validation using young and mature biofilms on implant materials.

The cadaver mouse subcutaneous implant infection model provided an intermediate step between *in vitro* testing and *in vivo* infection studies. All four BAG formulations were easy to apply and remained localized around the implant. In this model, BAG putty and cream alone achieved only modest reductions of CFU on the implant surface (approximately 2–3 log), whereas both SAAP-148-loaded formulations completely eradicated bacteria on implants and in surrounding tissue. Thus, the SAAP-148 peptide was essential for enhancing the antibacterial activity of BAG alone, resulting in a formulation capable of achieving infection clearance in this cadaver model.

The subsequent *in vivo* subcutaneous implant infection model was performed with a 1-day follow-up to specifically evaluate the rapid early antibacterial effect of SAAP-BAG putty. Although local pH was not measured *in vivo*, the *in vitro* data demonstrate the expected alkalinizing effect of BAG. Despite expected local alkaline local environment, no apparent adverse clinical signs were observed in the mice during the 1-day follow-up. Dedicated studies are required to evaluate local tissue tolerance and histocompatibility. BAG putty alone failed to clear infection from any implant (18/18 culture-positive), while SAAP-BAG putty achieved complete bacterial eradication from all implants (0/18 culture-positive). However, 7/18 surrounding tissue samples remained culture-positive, although CFU numbers were approximately 2-log lower. This result indicates that local peptide release at the current dose and formulation is sufficient for implant sterilization but does not fully eradicate bacteria in the surrounding tissue. The incomplete tissue clearance may be related to reduced availability or activity of SAAP-148 beyond the immediate vicinity of the formulation and/or limited accessibility of the peptide to tissue-associated bacteria. Previous work from our group using a similar subcutaneous implant infection model demonstrated that bacteria originating from titanium implants can be present in the surrounding tissue and associate with host cells, including macrophages (Riool et al., 2014), suggesting that tissue-associated bacteria may represent a less accessible bacterial reservoir. Future studies evaluating later time points to assess whether this tissue colonization persists, and if so what their spatial distribution is in relation to local concentrations of SAAP-148 could then help clarify the underlying mechanism. In such cases, combination with the standard systemic antibiotic prophylaxis may also contribute to clear the bacteria from the surrounding tissue.

This experiment was performed only with *S. aureus* and with BAG putty and BAG putty eluates in order to prioritize the putty as an already clinically used preparation, in order to minimize the number of animals. Further evaluation against *A. baumannii in vivo* is warranted to study efficacy also against this important pathogen in

tissue-based settings. In addition, further studies are needed to assess long-term efficacy, formulation-specific safety and histocompatibility, peptide release and clinical translatability. Although the present study used a subcutaneous rather than orthopedic implant infection model, the observed results pattern is comparable to murine orthopedic infection models. In such models local antimicrobial coatings or carriers often markedly reduce bacterial burdens on implants and in peri-implant tissues but rarely achieve complete eradication without systemic support (Kao et al., 2017; Nishitani et al., 2015; Wang et al., 2017; Wittmann et al., 2022). Therefore, the rapid early bactericidal activity of SAAP-148 may complement BAG by providing immediate local antibacterial protection during the initial post-application phase. Taken together, these findings support SAAP-loaded BAG putty as a promising local antimicrobial strategy for implant-associated infection prevention and treatment, with complete implant sterilization achieved in the *in vivo* model. Moreover, SAAP-loaded BAG cream, like the SAAP-BAG putty, also showed strong antibacterial activity *in vitro* and in the cadaver model, but remains an experimental formulation that should be evaluated in future *in vivo* studies. This study demonstrates that BAG S53P4 formulation design strongly influences antibacterial efficacy. Clinically used BAG putty showed limited intrinsic activity, whereas the experimental BAG cream was more active. Incorporation of SAAP-148 markedly improved antibacterial activity, eradicating multidrug-resistant planktonic bacteria and established biofilms *in vitro*, and *S. aureus* bacteria on implant surfaces in 1-day mouse cadaver and *in vivo* models. Thus, SAAP-loaded BAG formulations represent a promising translational approach combining rapid local antimicrobial activity with the clinically established bone-filling and later-stage antimicrobial properties of BAG S53P4.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by Animal Welfare Body of the Amsterdam UMC (location AMC), Amsterdam, the Netherlands. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

DR: Formal Analysis, Writing – original draft, Methodology, Investigation, Visualization, Conceptualization, Validation. PB: Writing – review and editing, Supervision, Validation, Conceptualization. Ld: Investigation, Writing – review and editing, Formal Analysis. MR: Formal Analysis, Investigation, Writing – review and editing, Conceptualization. SZ: Validation, Conceptualization, Project administration, Funding acquisition, Supervision, Writing – review and editing.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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